Exhibit 1

Structural and functional properties of *ras* proteins

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ABSTRACT

The *ras* proteins belong to a family of related polypeptides that are present in all eukaryotic organisms from yeast to human. Their extraordinary evolutionary conservation suggests that they have essential cellular functions, although their exact role remains unknown. Mutations in specific amino acids and overexpression of normal proteins have been linked to altered proliferation and/or differentiation and, particularly, to neoplastic processes. Mature *ras* proteins are located on the inner side of the plasma membrane, and their biochemical properties include binding and exchange of guanine nucleotides and GTPase activity. The favored hypothesis for *ras* function is that these proteins exist in an equilibrium between an inactive conformation (p21 · GDP) and an active conformation (p21 · GTP) in which they are able to interact with their as yet unknown cellular target or targets. Similarities in cellular location, structure, and biochemistry with other known regulatory (G) proteins suggest that they play a role in transduction of signals from the cell surface. The elucidation of the crystal structure of normal and transforming *ras* proteins and the identification of cellular proteins that interact directly with them (GAP, CDC25) or suppress some of their biological effects (Krev-1) have opened new avenues in the search for their elusive cellular targets and in the elucidation of the functional role of *ras* gene products. — Santos, E.; Nebreda, A. R. Structural and functional properties of *ras* proteins. *FASEB J.* 3: 2151–2163; 1989.

Key Words: ras proteins · oncogenes · malignant transformation · signal transduction · G proteins

THE *ras* GENES ARE MEMBERS OF A multigene family encoding closely related proteins whose amino acid sequences are highly conserved in evolution, which suggests that they have essential cellular functions. Point mutations in mammalian *ras* genes are responsible for expression of altered protein products capable of transforming NIH 3T3 cells to a malignant phenotype. Detection of activating mutations in a significant percentage of natural tumors indicates that *ras* oncogene products play a prominent role in neoplastic processes. Overexpression of normal p21 has also been linked to malignant transformation.

Ras proteins are synthesized in the cytosol and become associated with the inner side of the plasma membrane after posttranslational modifications. The biochemical properties of *ras* proteins include binding, exchange, and hydrolysis of guanine nucleotides. Normal and transforming *ras* proteins bind guanine nucleotides with similar affinities, but the intrinsic GTPase activity is impaired in transforming alleles of *ras* proteins. In addition, the GTPase activity of normal but not transforming mammalian *ras* proteins is specifically activated in vivo through interaction with a cellular protein (GAP). The current working hypothesis for *ras* function is that on GTP binding, p21 acquires an active conformation that enables it to carry out is functional effects by interacting with as yet unidentified intracellular target(s).

A great deal is known about the structure and function of *ras* proteins, as they are the first oncogene products for which the crystal structure is available. However, in spite of the detailed biochemical and structural characterization of the *ras* proteins, the functional role of the products of *ras* proto-oncogenes and oncogenes in physiological or pathological processes remains largely unknown and we are not yet able to define the site of action of the diverse *ras* proteins in the pathways controlling proliferation and/or differentiation.

The similarities of *ras* proteins to other nucleotide-binding regulatory proteins (G proteins) suggest that they play a role in signal transduction. The observation that *ras* proteins from organisms as distant as yeast and human are functionally interchangeable indicates that different biological systems in which *ras* genes have been characterized may be amenable to experimental analysis in order to answer the same fundamental questions about *ras* functions.

During the past couple of years, reviews have been published on the role of *ras* genes in normal and neoplastic processes (1) and on the participation of *ras* oncogenes in the etiology of human tumors and carcinogen-induced animal tumors (2, 3). Overviews on the *ras* genes of the

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yeast *Saccharomyces cerevisiae* (4) and on the GAP proteins (5) have also appeared. Here we deal primarily with the structure and function of the *ras* gene products and focus on recent findings related to the biochemistry, structure, and cell biology of these proteins that offer new insights into their possible functional role.

PRIMARY STRUCTURE

Ras genes appear to be ubiquitous in eukaryotic cells, and yeasts are the lowest organisms found to possess functional *ras* genes. The remarkable degree of conservation between species as far apart in evolution as yeast and human strongly suggests that *ras* gene products play a fundamental role in key cellular processes. The structure and chromosomal location of known *ras* genes and pseudogenes in mammalian and yeast cells have been reviewed (1, 4). In this section we focus on the primary structure of the *ras* proteins and related gene products. **Figure 1** presents an alignment of the amino acid sequences of these proteins. Taking the mammalian *ras* proteins as a paradigm, four domains can clearly be defined in the structure of these proteins (**Fig. 2**). The first one encompasses the first 80 amino acids and is almost completely conserved among all mammalian *ras* gene products. In this domain the activating point mutations have been found to occur in natural tumors. The *ras* genes of lower eukaryotes also show very high conservation (> 80%) in this domain (Fig. 1). The next 80 amino acids define a second domain that shows slightly less conservation (70–80% homology between any pair of vertebrate gene products) (Figs. 1 and 2). The rest of the molecule, except for the last four amino acids, constitutes the so-called hypervariable region, with sequences specific for each *ras* gene. It is reasonable to speculate that the conserved first two domains contribute a common "catalytic" function to all *ras* gene products and that the hypervariable region contributes to function and/or target specificity. The COOH-terminal four amino acids constitute a motif (CAAX box, where A is any aliphatic amino acid and X is any amino acid) that is highly conserved among all *ras* and *ras*-related genes (Fig. 1). This sequence is present not only in *ras* and *ras*-related proteins but also in the carboxyl terminus of other unrelated proteins, including the α and γ subunits of several G proteins, nuclear lamins, and unprocessed yeast alpha mating factor (6).

Posttranslational modifications

The cysteine residue in the CAAX box is required for acylation, membrane localization, and transforming activity of *ras* proteins (7). It is important because it is the target for various posttranslational modifications that have important functional consequences (6–9). Cys186 is acylated through a thioester linkage with palmitic acid (7) and is also required for an essential earlier processing event, which in the case of yeast is under control of the *DPR1/RAM* gene (4). There is evidence to suggest that this earlier processing in yeast involves proteolytic removal

of the last three amino acids, placing the cysteine at the carboxyl terminus (4). Such proteolytic modification would be consistent with the existence of previously unexplained, nonacylated, intermediate forms of p21 (7). Initial support for such proteolytic processing in mammalian cells was provided by the detection of yet another modification of the COOH-terminal sequences: carboxymethylation, probably at the α-carboxyl of the COOH-terminal cysteine (6). Recently, the occurrence of both carboxymethylation and COOH-terminal proteolysis has been confirmed in rodent cells that express high levels of *ras* proteins (8). The order and extent of these posttranslational modifications are still obscure. In fact, only a subpopulation of mature membrane p21 molecules appears to be acylated (7) and the stoichiometry of carboxymethylation is unclear (6, 8, 9).

The modifications outlined above, which at least in the case of the palmitylation are reversible (9), offer the potential for dynamic regulation of *ras* protein localization and function. An attractive model suggests that the three-amino-acid extension after Cys186 could constitute a signal sequence that allows transport of the proteins to appropriate subcellular sites where proteolytic activation could make the proteins convenient substrates for acylation or carboxymethylation. Such a two-step modification mechanism would ensure proper membrane trafficking and targeting of *ras* proteins within the cell. A corollary of this model is that membrane localization does not necessarily require acylation. In fact, it may be exactly the other way around. Evidence in this sense is provided by the significant percentage of membrane *ras* that is not acylated (6, 7) and by the observation that mutation at Cys186 (the target for palmitylation) blocks a processing step preceding acylation (8). The functional significance of the COOH-terminal CAAX box was evaluated in recent experiments in which an amino-terminal myristoylation signal was fused to nonpalmitylated *ras* proteins in which the critical Cys186 had been replaced by serine. This modification regenerated appropriate membrane localization and transforming activity to activated (Leu61) proteins and, interestingly, also conferred transforming properties to otherwise normal (Gln61) *ras* proteins (10).

Ras-related genes

Relaxed hybridization and/or use of specific oligonucleotide probes has allowed the detection of new classes of *ras*-related genes in different eukaryotic organisms (Fig. 1). These findings make it evident that *ras* genes are only one family within a wider superfamily of related genes. In Fig. 1 we present an alignment of newly identified sequences in relation to a prototype H-*ras* protein and offer a tentative grouping of the best-paired sequences within different *ras*-related gene families (Fig. 1). These families, like the *ras* family, are conserved throughout evolution in many eukaryotic organisms. Thus, *rho* genes, initially identified in *Aplysia* (1), have also been found in humans (11, 12) and yeasts (13). Other gene families have so far been found only in mammalian cells—for example, R-*ras* (14) (detected in humans and rodents) and *ral* (15)
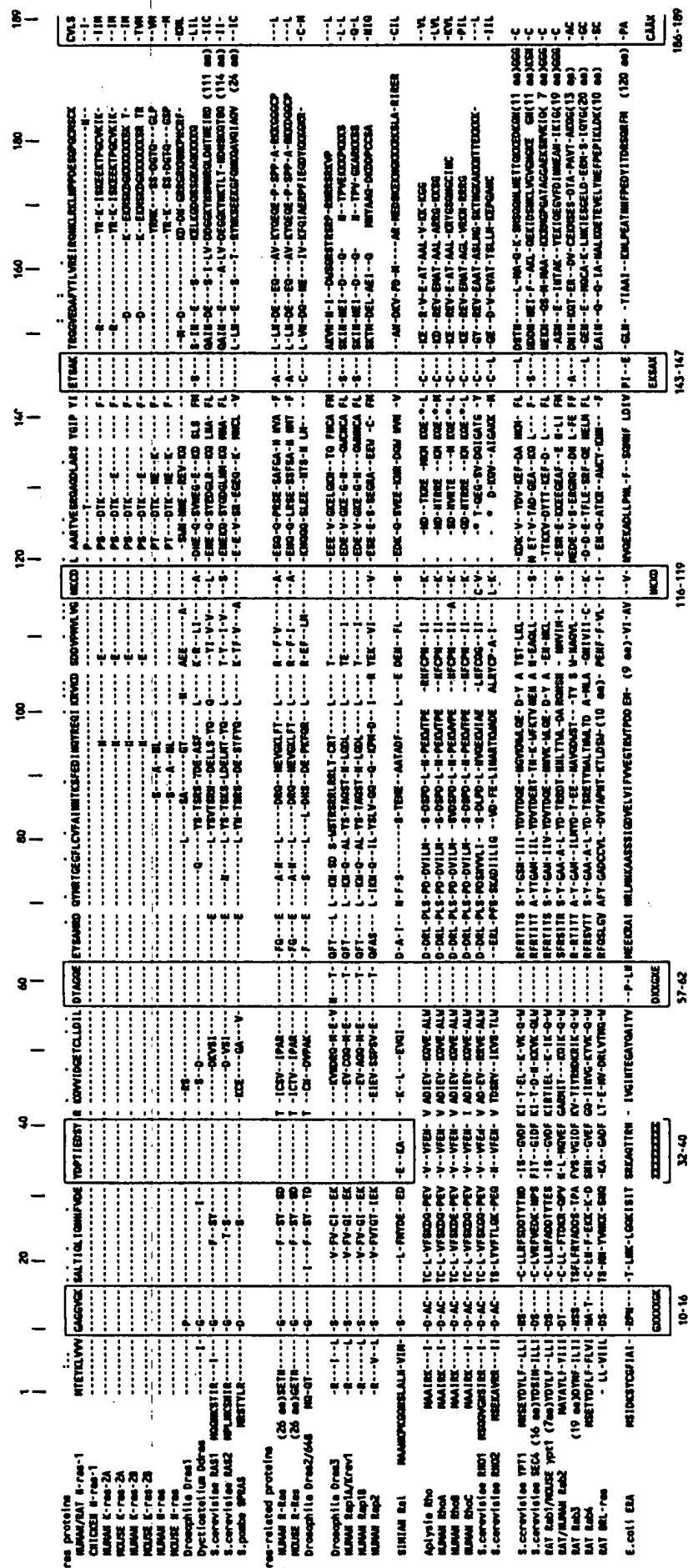
Figure 1. Primary sequence of *ras* and *ras*-related gene products. All proteins are compared here with H-*ras* proteins. Blank spaces and deletions (* = 17 residues) are introduced for optimal alignment. Identical residues are indicated by a dash. Boxes indicate conserved regions involved in posttranslational modifications (CAAX box), effector regions (32-40), and nucleotide interactions (all others). Double and single dots outside box areas indicate, respectively, residues conserved in all or more than 75% of the sequences presented.

A. EVOLUTIONARY CONSERVATION

B. FUNCTIONAL DOMAINS
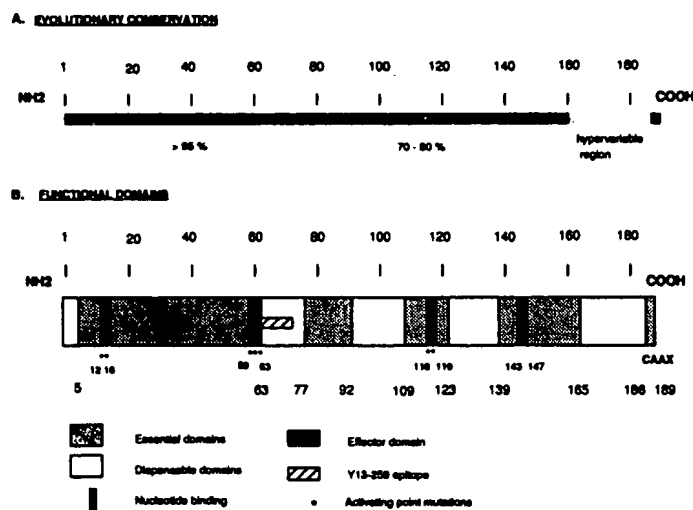
Figure 2. Structural and functional domains of mammalian *ras* proteins. *A*) Percentage of evolutionary conservation of different regions of the *ras* proteins. The numbers under *B* indicate amino acid residues defining the boundaries of important domains (marked by the boxes) within the primary structure of these proteins.

(detected in primates and humans). Another *ras*-related gene, *Dras3* of *Drosophila* (1) has been used to uncover the human *rap1* and *rap2* genes (16, 17). Interestingly, *rap1* is the same gene as *Krev-1*, which was independently identified by its ability to reverse the transforming activity of *ras* oncogenes (18). Finally, the yeast YPT1 gene appears to be more related to *rab1* than to *rab2, 3,* and *4,* which are *ras*-related genes identified in a cDNA library of rat brain (19, 20). Other genes included in this family are rat *BRL-ras* (21) and human *rab2* (22). The recently identified *ara* gene from the plant *Arabidopsis* (23) appears to fall in this group too. *Drosophila Dras2,* considered generally to be a genuine *ras* gene, appears to us to be closer to R-*ras* than to the *ras* prototype and therefore has been classified as a *ras*-related gene in Fig. 1. Although all members of the superfamily show an extraordinary conservation of sequences dedicated to guanine nucleotide binding (10-16, GXXXXGK; 57-62, DXXGXE; 116-119, NKXD; and 143-147, EXSAX), only some of them show significant conservation of the so-called effector region (32-40) of *ras* proteins (Fig. 1). The sequences recognized by monoclonal antibody Y13-259 (63-73) appear to be present only in *ras* genes and not in *ras*-related genes (Fig. 1), suggesting that reactivity with this antibody may be used as an operational definition for authentic *ras* proteins as opposed to *ras*-related proteins.

It is likely that each *ras*-related gene family has a specific functional role. Yeast *rho* proteins are clearly involved in a totally different biochemical pathway than the *ras* proteins (13). The fact that some of these *ras*-related families (R-*ras, rap*) conserve the effector region, while others (*rho, rab*) do not (Fig. 1), suggests that their functions may in some cases be complementary to those of *ras* genes and in other cases totally different. The conservation of both the nucleotide-binding regions and the effector region in *rap1/Krev-1* suggests that its antitransforming activity may result from competition with *ras*

oncogene products for the same intracellular pool of guanine nucleotides and/or effectors.

Other nucleotide-binding proteins

The structural homology between *ras* proteins and other nucleotide-binding proteins such as the α subunits of G proteins, transducin, bacterial EF-Tu, and other enzymes such as adenylate kinase, phosphofructokinase, or tubulin was noted long ago (1). A survey of known guanine nucleotide-binding proteins showed in all of them the conservation of three consensus elements (GXXXXGK, DXXG, and NKXD) with a distinct spacing between them (24). As seen in Fig. 1, those three consensus sequences, plus EXSAX (143-147), are required for nucleotide binding in all *ras* and *ras*-related proteins. However *ras* and *ras*-related genes share other areas of homology outside those regions (Fig. 1). Therefore, in order to classify a new gene as *ras*-related, additional homology besides that including the consensus G-binding areas is needed. In this regard, the ERA protein (25) from *E. coli* (Fig. 1) lacks sufficient homology to be considered a member of a *ras*-related family.

FUNCTIONAL MAPPING OF *ras* PROTEINS

Mutational analysis and comparison with partially related proteins whose structure/function features are known have made it possible to draw a detailed map of functional domains within the primary sequence of *ras* proteins (Fig. 2). This mapping allows us to define essential and dispensable domains for transforming activity as well as regions involved in nucleotide binding and interactions with cellular effectors such as GAP or a variety of neutralizing and nonneutralizing antibodies.

Nucleotide-binding regions

The homology of *ras* proteins with guanine nucleotide-binding proteins (1, 24) allowed an initial identification of some sequences involved in nucleotide binding. The crystallographic data recently obtained (26, 27), in conjunction with mutagenesis studies involving deletions or substitutions of specific amino acids (reviewed in 1), have allowed a detailed identification of different domains involved in GTP/GDP binding (Figs. 1 and 2). Most mutants defective in binding or hydrolysis of guanine nucleotides are also deficient in transforming ability (1). Interestingly, two mutants in amino acids 116 (Asn-Ile) and 119 (Asp-Ala) prevent any measurable binding of GTP while simultaneously activating the transforming potential of otherwise normal (Gly12, Gln61) p21 *ras* proteins (1).

The neutralizing antibody Y13-259 (epitope: 63-73) inhibits GTP/GDP exchange but not binding or hydrolysis of guanine nucleotides (28). Because this epitope is not in direct contact with the bound nucleotides, it appears that this effect and the neutralizing effect are due to indirect conformational changes resulting from binding of the antibody.

Essential and dispensable domains: the effector domain

Analysis of deletion mutants has defined five noncontiguous domains (residues 5–63, 77–92, 109–123, 139–165, and 186–189) that are absolutely necessary for *ras* transforming function (Figs. 1 and 2). The areas outside those domains (1–5, 64–76, 93–108, 124–138, and 166–185) can be deleted without affecting significantly the transforming ability of *ras* proteins and are therefore designated dispensable domains (1). Most of the essential domains are involved in interaction with guanine nucleotides. However, changes of amino acids in region 32–40 that do not affect guanine binding or hydrolysis completely eliminate the transforming properties of oncogenic *ras* proteins (1). This suggested that this domain was necessary for interaction with a putative cellular target, and therefore this region has been called the effector domain (Figs. 1 and 2). Direct proof for this view has been provided by the recent demonstration that the GAP proteins interact with this domain (see The GAP proteins).

It is interesting to note that all essential domains lie in internal hydrophobic regions (β-sheet core) except for the effector domain, which is located in the surface of the molecule and is accessible to interaction with other cellular proteins such as GAP. Similarly, all dispensable domains are located on the external surface (see below).

3-DIMENSIONAL STRUCTURE

Until recently, our knowledge about the three-dimensional structure of *ras* proteins was based on computer predictions of secondary structure and on models based on the structure of partially homologous proteins such as EF-Tu (1). The recent crystallization of the "catalytic" domain (lacking the COOH-terminal 18 amino acids) of normal (Gly12) and oncogenic (Val12) H-*ras* p21 · GDP (26, 27) represents the first atomic description of any proto-oncogene or oncogene product and provides direct understanding of the structure/function aspects of *ras* proteins. We hope that the crystallization of complete *ras* proteins in the GTP form will follow soon. A schematic representation of the spatial interactions between regions of functional importance is presented in **Fig. 3**. The structure of *ras* proteins consists of six-stranded β sheets, four α helixes, and nine connecting loops (Fig. 3). The overall topology is considered that of an alternating α/β domain similar in general to that of other nucleotide-binding proteins like EF-Tu, although with some minor differences. The essential regions for *ras* function (Fig. 2) include all strands of the central β sheet as well as a few connecting loops and adjacent helixes. The crystal structure reveals that all looped regions are exposed on the surface. Four of those loops are involved in interactions with guanine nucleotides, another corresponds to the putative effector region, and still another corresponds to the binding site for the neutralizing antibody Y13-259 (Fig. 3). It is likely that other loops and exposed α helixes may be important for recognition by as yet unknown regulatory or effector molecules. The three "hot spots" where single amino acid substitutions can affect the transforming properties of *ras* genes (see *ras* oncogenes and
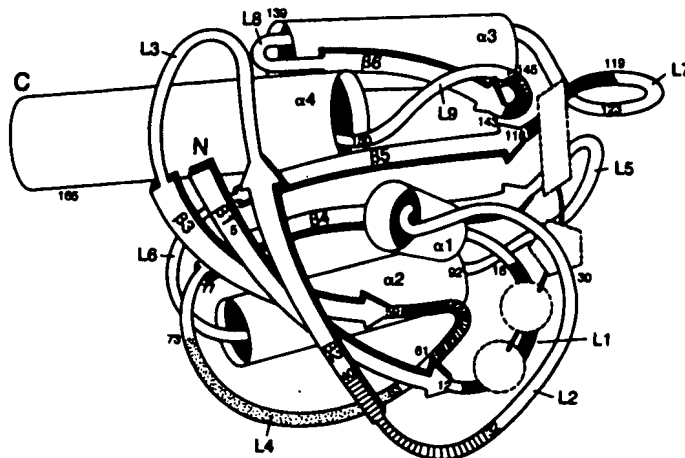
Figure 3. Backbone structure of H-*ras* proteins. Cylinders, arrowed ribbons, and tubes represent α helices, β strands, and loops, respectively. The GDP molecule is drawn with a broken line. The drawing is not to scale and numbers indicate only approximate positions of residues of interest. Stippled, lightly hatched, and densely hatched areas represent, respectively, Y13-259 epitope, effector region, and regions involved in nucleotide interaction and transforming ability.

carcinogenesis) are all located near the bound guanine nucleotide. Specifically, residues 10–16 (loop 1) and 59–63 (loop 4) are in the vicinity of phosphate groups. Residues 116–119 (loop 7) and residues 145–147 (loop 9) form a side of the pocket for the guanine base. Residue 30 (loop 2) is near the ribose sugar. Residues 59–63 (loop 4) are not in contact with the phosphate groups of GDP but are in direct contact with loop 1. It is possible that mutations at this position activate p21 through indirect conformational changes of loop 1. Furthermore, although *ras*-GTP crystals are not yet available, it appears that the position of residues 59–63 would correspond to that of the γ phosphate in the GTP-bound form; thus, mutations in that position might also directly affect GTP binding or GTPase activity. Residues 63–76 constitute a dispensable region in loop 4 that encompasses the epitope (63–73) of the neutralizing antibody Y13-259 (1). The overall structure of transforming (Val12) proteins is similar to that of the normal protein and their structural differences are localized to specific areas within the molecule (26, 27). The major difference is that loop 1 (which binds the β phosphate) is enlarged in the transforming *ras* proteins. The fact that the loop conformation around this phosphate in normal p21 is much tighter than that of p21 (Val12) may explain the reduced GTPase of the mutant proteins. This loop probably straddles the phosphodiester bond between the β and γ phosphates of GTP and therefore is the prime candidate to be the catalytic site for GTP hydrolysis in the normal p21 protein.

Quaternary structure

Experiments using radiation inactivation suggest that *ras* proteins exhibit a homo-oligomeric structure (most likely dimers or trimers) (29). A similar homo-oligomeric size has also been reported for the related *rab3* proteins (30). This quaternary structure clearly distinguishes *ras* proteins from the monomeric α subunits in classical G pro-

teins (αβγ heterotrimers) and establishes similarities with other homo-oligomeric proteins (such as EF-Tu, SV40 large T antigen, and *E. coli* CRP) that acquire their active conformations through subunit reorientation after nucleotide binding. This structure would be consistent with a mechanism of p21 activation based on rearrangement of the monomers within the oligomer as a result of guanine nucleotide binding (29). This model can also explain the transforming activity of mutants in residues 116 and 119 that lack the ability to bind guanine nucleotides (1). By analogy with the *crp** mutants of CAP (29) (which lack cAMP binding activity but are constitutively activated by the mutation through constitutive rearrangement of the monomers within the dimer), those mutations might cause a permanent reorientation of the monomers within the *ras* homo-oligomer.

BIOCHEMICAL PROPERTIES

The biochemical properties of *ras* and *ras*-related proteins include binding, exchange, and hydrolysis of guanine nucleotides (1). The GTPase activity of transforming proteins is severely impaired as compared with that of normal proteins (1). The amino acid sequence, biochemical properties, and subcellular location of *ras* proteins are similar to those of G proteins involved in transmembrane signaling through generation of second messengers. These considerations support the idea that *ras* function is regulated through conformational changes that depend on the type of bound guanine nucleotide (**Fig. 4**).

Nucleotide binding and hydrolysis

The domains of *ras* proteins involved in interaction with guanine nucleotides have been defined in detail in previous sections. Amino acids in regions 10–16 and 59–63 appear to interact with phosphate groups, while amino acids in region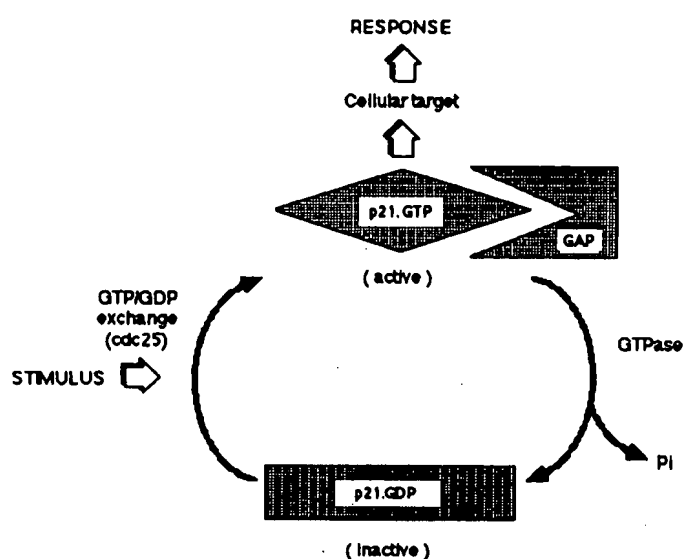s 116–119 and 143–147 form the pocket for the guanine base. All activating mutations detected in vivo are located around amino acids 12 and 61 and they appear not to affect significantly the binding affinity of the mutated proteins. However, in all cases those activating mutations cause a significant decrease of the intrinsic GTPase activity. Curiously, the decrease in intrinsic GTPase activity does not correlate with the transforming potential of the mutated proteins (1). Other mutations artificially introduced and not yet detected in vivo can also affect the binding affinity significantly. Thus, the substitution Lys16-Asn16 and a variety of substitutions in regions 116–119 and 143–147 produce drops in affinity that vary from several orders of magnitude to below detection levels (1). These substitutions that reduce binding affinity do not necessarily diminish the transforming ability of the mutated proteins; in fact, in some cases they activate the transforming potential of otherwise normal (Gly12, Gln61) *ras* proteins (1). Since the intracellular concentration of GTP is in the millimolar range (25:1 GTP/GDP), it is possible that these low-affinity, mutated proteins are still able to bind GTP in vivo. Alternatively, in cases where the mutation appears to cause total loss of binding (Asn116-Ile116, Asp119-Ala119) (1), it is possible that the mutation produces a conformational change similar to that produced by GTP binding (29).

The GAP proteins

As discussed above, there is an important quantitative discrepancy between decrease of intrinsic GTPase activity and transforming potential of mutated *ras* proteins (1). The recent discovery of a GTPase-activating protein (GAP) has provided a solution to this apparent paradox (5). This protein stimulates the GTPase activity of normal *ras* by more than 100-fold with no effect on the GTPase activity of oncogenic *ras* proteins. Analysis of various mutants reveals that the interaction between GAP and *ras* proteins occurs at the level of the effector region (amino acids 32–40) of *ras* proteins (5).

Bovine and human GAPs have been purified as single polypeptides of approximately 120 kDa and the corresponding cDNA clones have been isolated. A second form of GAP not expressed in adult tissues appears to be generated by differential splicing in humans. It should be noted that oncogenic forms of *ras* p21 in their GTP forms also bind GAP, although their GTPase activities are not activated (5). Interestingly, it appears that the lipid microenvironment is an important regulatory component of *ras*-GAP interaction. GAP activity is inhibited by various membrane lipids (phosphatidic acid, inositol phospholipids, and arachidonic acid) whose metabolism is altered during mitogenic stimulation (31).

The question that arises now is whether GAP is simply an upstream regulator of *ras* activity or the downstream target. The need for interaction of GAP with oncogenic *ras* proteins in order to achieve malignant transformation would argue for the latter possibility. However, the fact that the same GAP molecule interacts with different *ras* and *ras*-related proteins suggests that GAP may be a regulatory protein common to a superfamily of proteins.

RESPONSE

⇧

Cellular target

⇧

p21.GTP

GAP

(active)

GTP/GDP exchange (cdc25)

STIMULUS ⇨

GTPase

PI

p21.GDP

(inactive)

Figure 4. Biochemical model of *ras* function. See text for details. The existence of *cdc25*-like molecules in cells other than yeast is still hypothetical.

The 32–40 stretch is conserved in all *ras* genes and also in some *ras*-related genes such as R-*ras, rap,* and *ral* (Fig. 1), suggesting that different proteins interact with the same GAP. There is direct biochemical evidence for the interaction of the same GAP protein with H-*ras,* N-*ras,* and R-*ras* (5, 32). As expected from its lack of the effector sequences (Fig. 1), the GTPase activity of *rho* proteins is activated by a different protein of 29 kDa, *rho* GAP (33).

Recent studies point to the COOH-terminal region of *ras* proteins as a possible effector domain. R-*ras* conserves the effector region but does not transform cells (32). Studies of chimeric proteins between H-*ras* and R-*ras* genes indicate that the COOH-terminal portion of R-*ras* prevents this molecule from having typical *ras* transforming ability (32). Similarly, the determinants for G$_s$ activation of adenylate cyclase appear to lie in the COOH-terminal region of this molecule. These types of indirect evidence support the speculation that the 32–40 region, through interaction with GAP, might be involved in transmitting the GTP signal to the COOH-terminal portion of the *ras* proteins. This COOH-terminal region is specific for each member of the *ras* superfamily (Fig. 1) and would provide for specific protein-protein interaction with distinct cellular targets to produce specific biological effects.

GDP/GTP exchange

The biochemical steps involved in GDP/GTP exchange and their physiological implications are only now beginning to be fully understood. The specific inhibition of this exchange by the neutralizing antibody Y13-259 (28) is indicative of its functional importance. The dramatic increase in the rate of exchange of GDP for GTP observed in the presence of low Mg^{2+} concentrations indicates that removal of this divalent cation produces conformational changes that enhance the affinity for GTP (1).

Recent work with yeast suggests that the product of *CDC25* is the factor regulating exchange of GDP for GTP through direct, transient interaction with *ras* proteins. Disruption of *CDC25* is lethal in yeast, but the lethality can be suppressed by activated *ras* proteins (4, 34). Mammalian *ras* proteins can also interact with CDC25 (34), and it is therefore likely that a protein functionally similar to the CDC25 protein exists in mammalian cells. This would help explain mammalian *ras* mutants in which a Ser17-Asn17 substitution causes preferential affinity for GDP and inhibition of NIH 3T3 cell proliferation (35). A mechanism could be envisioned where the mutant protein locked in the GDP state depletes the pool of CDC25-like molecules needed to interact with the endogenous *ras* proteins to promote proliferation. The fact that activated *ras* proteins overcome that inhibition (35) suggests that they can bypass the requirement of CDC25 because they stay preferentially in the GTP state.

Biochemical model for *ras* function

The current model for *ras* function predicts that the *ras* proteins exist in an equilibrium between an active (p21 · GTP) and an inactive (p21 · GDP) state. Upon GTP binding, p21 acquires its active conformation and is thus able to interact with its target molecule(s) (Fig. 4). The state of this equilibrium is the result of the interaction of intrinsic biochemical parameters of the p21 molecule with external factors. Intrinsic parameters are the concentration of *ras* proteins, their relative affinities for GTP and GDP, the GTP/GDP exchange rate, the intrinsic GTPase activity, and other conformational changes caused by specific mutations that may affect intrinsic biochemical properties. External factors include GAP, Mg^{2+} concentration, external kinases that specifically phosphorylate the *ras* proteins, CDC25-like molecules, and possibly other as yet unknown regulatory proteins. We favor a model in which GAP facilitates both interaction of the *ras* · GTP complex with the downstream target and subsequent deactivation to the *ras* · GDP form. CDC25-like molecules would be needed to couple external stimuli to *ras* activation (Fig. 4).

For normal *ras* proteins the equilibrium would be displaced toward the inactive state. Oncogenic mutations would cause *ras* proteins to stay preferentially in the active conformation and thus produce a continuous flow of signals resulting in cellular transformation (Fig. 4). The experimental evidence indicates that decrease of the GTPase activity (resulting from mutations around amino acid 12 or 61) is the preferential mechanism of activation of oncogenic *ras* proteins. In cases where the nucleotide-binding ability is negligible (Ile116 and Ala119), the homo-oligomeric structure of *ras* proteins and the functional analogy with crp* mutants suggest that those amino acid substitutions produce a constitutive conformational activation mimicking that produced by GTP binding (29) (see Quaternary structure).

BIOLOGICAL PROPERTIES

Unveiling the role of cellular *ras* genes and their products in physiological and pathological processes can be greatly helped by models simpler than mammalian cells. Although results may not be directly extrapolated from one organism to another, the study of *ras* products in organisms more amenable to experimental manipulation may shed some light on their general mechanism of action. In this section we describe the biological properties of *ras* proteins in different biological systems ranging in complexity from yeast to human.

Lower eukaryotes

The use of yeast as a model makes it possible to use powerful genetic and biochemical analyses that are not available in mammalian cells (4). *Saccharomyces cerevisiae* contains two *ras* proteins, RAS1 and RAS2 (309 and 322 amino acids, respectively), whose large size is due to seven extra amino-terminal amino acids and a large COOH-terminal region that is not conserved in their mammalian counterparts (Fig. 1). Disruption of either the *RAS1* or *RAS2* gene has no effect on growth on fermentable carbon sources, although *ras2⁻* cells are not able to grow on non-

fermentable carbon sources. Thus, neither *RAS1* nor *RAS2* is by itself an essential gene. However, *ras1⁻ ras2⁻* cells are nonviable and apparently are blocked in the G1 phase of the cell cycle. These properties indicate the relevance of *ras* genes in normal cell proliferation. The argument that the study of yeast *RAS* genes may contribute to our understanding of the function of mammalian *ras* genes is based on the partial functional interchangeability shown by both yeast and mammalian gene products. Thus, *1*) yeast and mammalian *ras* proteins have similar biochemical properties; *2*) *ras* proteins with mutations in positions analogous to those that activate mammalian *ras* proteins exhibit decreased GTPase activity and cause dramatic changes in the physiology of yeast cells (4); *3*) mammalian *ras* genes can replace yeast genes for yeast growth; and *4*) the NH_2-terminal portion of yeast genes can substitute for the corresponding portion of mammalian *ras* genes to induce transformation of NIH 3T3 cells provided that activating mutations are introduced into the yeast genes. Although these observations point to basic similarities, there are also obvious differences. First, the complementation of yeast growth by intact mammalian *ras* proteins is inefficient, and the intact yeast protein cannot transform NIH 3T3 cells unless its COOH-terminal portion is deleted (4).

The exact function of yeast *ras* proteins remains unknown, but genetic and biochemical analyses show that they regulate cell growth by acting as positive regulators of adenylate cyclase (1, 4). The mechanism of this stimulation is unknown, but it is unlikely that *ras* proteins are the direct regulatory subunits of the yeast adenylate cyclase. It appears rather that they are important members of a cascade of regulatory proteins whose final effect is stimulation of the catalytic subunit. Other proteins, such as the product of the *CDC25* gene, act upstream of *ras* proteins, and in fact there is strong evidence that the *CDC25* gene product is the exchange factor regulating the binding of GTP to *ras* (34). Other proteins, such as the products of *GPA1* and *GPA2*, might somehow be modulated by the *ras* proteins and would act downstream from *ras* proteins as the actual G proteins of the adenylate cyclase (36).

Regarding the function of *ras*-related genes, the recent report demonstrating that yeast *SEC4* and *YPT1* genes and probably their mammalian counterparts are involved in control of the cellular secretory machinery is of great interest (37).

Despite the evidence in *S. cerevisiae*, *ras* genes and adenylate cyclase are not functionally linked in any other eukaryotic organism studied, including fungi close in the evolutionary scale, such as the fission yeast *Schizosaccharomyces pombe*. Disruption of the *SPRAS* gene in this fungus does not affect cell growth or cAMP levels, but it interferes with sexual differentiation by blocking the mating ability of this yeast and causing inefficient sporulation. It is interesting that a gene (*byr*) that suppresses some of the phenotypic changes produced by disruption of the *SPRAS* gene is a protein kinase (38). This suggests that SPRAS acts, at least in part, as a modulator of protein phosphorylation. There is no indication yet as to what biochemical or signaling pathway is regulated by SPRAS.

Ras genes have also been studied in organisms such as slime molds, sponges, insects, amphibians, and fish. In most cases the available evidence indicates that *ras* proteins participate in transduction of proliferative signals (1). Interestingly, in *Drosophila melanogaster*, the expression of an activated *Dras2* gene (Gly14-Val14, position equivalent to mammalian Gly12) caused important developmental abnormalities in transgenic flies (39), suggesting a role of *Dras* genes not only in cell proliferation but also in differentiation.

The work on *ras* with *Xenopus* oocytes has been particularly informative about possible roles of *ras* proteins in the cell cycle. Fully grown *Xenopus* oocytes are arrested at the G2/M border of the first meiotic division and progesterone or insulin releases the G2 block, leading to meiotic maturation involving germinal vesicle breakdown. Microinjection of *ras* proteins also induces meiotic maturation without affecting cAMP levels (1). These observations indicate that *ras* proteins are not involved in the adenylate cyclase pathway in this organism. On the other hand, the neutralizing *ras* antibody Y13-259 specifically inhibits maturation by insulin but not by progesterone, suggesting a role of the *ras* proteins in the insulin pathway (40). The observation that microinjected *ras* proteins are able to induce maturation in the absence of protein synthesis (41) is also of great interest in light of the identification of important components (*cdc2*, MPF, etc.) of the regulatory machinery of the cell cycle that are conserved in yeast, amphibian, and mammalian cells (42).

Mammalian *ras* genes

The role of mammalian *ras* proteins is still poorly understood. However, it is increasingly apparent that *ras* gene products play a role not only in cell proliferation but also in differentiation of mammalian organisms. The following sections focus on different aspects of mammalian *ras* proteins that are relevant to their functional role.

Ras *and signal transduction*

Cellular location and structural and biochemical similarities to G proteins suggest that *ras* proteins participate in signal transduction. A direct relation of *ras* proteins and the adenylate cyclase system of mammalian cells has been rejected (1) and the most recent studies have focused on a possible role of *ras* proteins in the phosphoinositide (PI) signaling pathway. Early studies suggested that *ras* proteins mediated stimulation of either phospholipase C (PLC) or phospholipase A_2 (PLA_2) (1). In contrast, other reports described inhibition of both PLC and PLA_2 in *ras*-transformed cells (reviewed in 44). Studies of the PI pathway in normal cells microinjected with specific *ras* antibodies (43) or in transformed cells (44) clearly demonstrate that *ras* proteins are not direct regulatory elements of either PLC or PLA_2. Interestingly, transformation by *ras* produced specific changes in PI metabolism (activation of PLA_2 and uncoupling of PLC from external signals), but the same changes are produced by transformation

by membrane-associated and cytoplasmic oncogenes (44). These observations rule out a specific role of *ras* proteins as regulatory elements of PLC or PLA$_2$, the main effectors in the PI signaling pathway.

Common biochemical pathways for proliferative signal transduction

Important clues to the functional role of *ras* proteins have been obtained by microinjection of the neutralizing antibody Y13-259. Microinjection of the antibodies causes transient reversion of *ras*-transformed cells and of cells transformed by tyrosine kinase, membrane-associated oncogenes such as *fms*, *src*, or *fes*. However, the antibody has no effect on cells transformed by cytoplasmic oncogenes with serine or threonine kinase activity (*mos* and *raf*) (1). These experiments suggest that *ras* constitutes a bottleneck, transducing proliferative signals that originate in a variety of surface receptors and/or membrane-associated molecules or their altered (oncogenic) versions. The *mos* and *raf* genes might act in totally separate pathways or in the same pathway, downstream from *ras*. The observation that transformation by *raf* and *mos* produces changes of PI metabolism similar to those produced by transformation by membrane-associated oncogenes (44) suggests that these gene products are located downstream from *ras* in a common biochemical pathway conveying proliferative signals in normal and transformed cells (Fig. 5). Further support for this model is provided by the report that transformation by *ras* and other membrane-associated oncogenes or surface stimulation with growth factors results in phosphorylation of Raf-1 and activation of its serine/threonine kinase activity (45). Experiments on the participation of *mos* in meiotic maturation in *Xenopus* oocytes are also consistent with *mos* acting downstream from *ras* in a common pathway inducing meiotic maturation (46).

The participation of *ras* products in conveying mitogenic signals from surface to nucleus is also supported by studies of transcriptional activation in transformed cells (47). Transformation mediated by H-*ras* results in activation of specific, *jun*-like, transcriptional factors (PEAI)

Figure 5. Common pathways of signal transduction. Based on evidence discussed in the text, the indicated oncogenes are proposed to participate in a common pathway, transducing signals from cell surface to nucleus. *Ras* would allow integration of different extracellular signals into this common pathway.

which are also activated by phorbol esters and serum. However, this effect is not limited to *ras* since other transforming oncogenes (membrane, cytoplasmic), but not immortalizing oncogenes (nuclear), are able to activate the same transcriptional factors (47).

These observations suggest the existence of common pathways for transmission of the mitogenic signal from surface receptors to nuclear transcriptional factors. It appears that *ras* acts at a pivotal step in this pathway, allowing the integration of different surface signals into the common pathway (Fig. 5). The sequence similarities observed between GAP (5) and the nonreceptor tyrosine kinases such as *src* or *crk* products (48) suggest the possible existence of a common regulatory protein(s) allowing such an integration.

Ras *expression*

Mammalian *ras* genes are expressed in all cell lineages and organs, although there are differences in expression through pre- and postnatal development and certain adult tissues preferentially express one member of the family over another (49, 50). Although increased *ras* gene expression has been described in actively proliferating tissues such as regenerating liver (1), it does not necessarily correlate with active proliferation. Studies of RNA levels in rodents (49) and immunocytochemical analysis of *ras* proteins in fetal and adult human tissues (50) led to similar conclusions. Detectable levels of *ras* gene products were present in all tissues analyzed. In some cases, immature proliferating cells contained more *ras* products than mature cells. In others, certain differentiated cells such as neurons of the brain, epithelial cells of endocrine glands, or muscle cells express very high levels of p21. These patterns of expression are consistent with the idea that H-, K-, and N-*ras* gene products have different functions and may be required in different amounts at different times in a tissue-specific manner. Also, they indicate that the role of *ras* genes is not restricted to cell proliferation, as terminally differentiated tissues such as brain have the highest level of *ras* proteins of any human tissue (50).

Ras *and differentiation*

A role of *ras* proteins as signal transducers can be compatible with effects not only in control of cell proliferation and malignant transformation but also in positive or negative control of differentiation processes. Transforming (but not normal) *ras* genes are able to induce terminal differentiation of the rat pheochromocytoma cell line PC12, thus promoting neurite outgrowth and neuronlike morphology (1). Nerve growth factor (NGF) and cAMP are physiological inducers of such differentiation, apparently through different biochemical pathways. Injection of antibody against *ras* proteins inhibits differentiation mediated by NGF but not by cAMP when injected into PC12 cells (1), suggesting important similarities between the differentiation processes induced by *ras* and by NGF.

Myogenic differentiation is blocked by constitutive expression of activated *ras* oncogenes (51). The transition
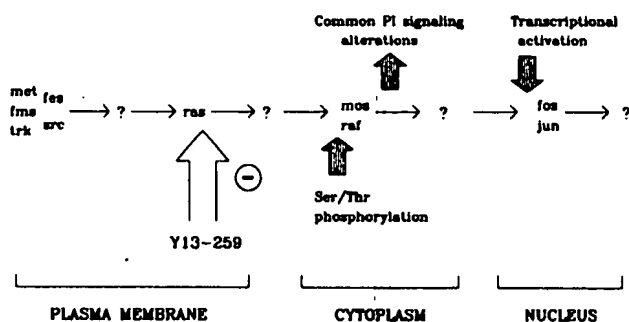
of myoblasts to terminally differentiated myotubes in culture is inhibited by transforming growth factor β (TGFβ) and fibroblast growth factor (FGF) through mechanisms independent of cell proliferation. The inhibition of myogenic differentiation by *ras* does not require cell proliferation and affects an early step of the differentiation program. It appears that *ras* blocks myogenic differentiation by preventing accumulation of regulatory factors required for transcriptional induction of muscle-specific genes (51). In light of the similarities, it is possible that the same intracellular signals associated with TGFβ or FGF are amplified in myoblasts bearing activated *ras* genes. Other tyrosine kinase oncogenes such as *src* and *erbB* are also able to induce differentiation in PC12 cells or to block myogenic differentiation. This observation provides additional evidence for the participation of tyrosine kinase oncogenes and *ras* proteins in common signal transduction pathways (Fig. 5) having similar effects on different differentiation programs.

Interactions of *ras* gene products with other differentiation systems have also been described. Thus, *ras* oncogenes transform erythroid cells ·without affecting their differentiation program (1) and also induce malignant transformation and plasmacytoid differentiation of human lymphoblasts (52). Also, while primary granulosa cells transfected with SV40 lose their ability to produce progesterone, double transfectants with SV40 and H-*ras* oncogene produce progesterone at levels comparable to those of differentiated primary cells, suggesting that *ras* oncogenes play a role in preserving inducible steroidogenesis (53). Finally, *ras* alone or in combination with other oncogenes appears to interfere with the differentiation of mammary epithelial cells (54).

ras oncogenes and carcinogenesis

ras genes can be malignantly activated through qualitative or quantitative mechanisms (1, 2). The most frequent mechanism of activation involves point mutations that affect the interaction with guanine nucleotides. Mutations in naturally occurring oncogenes have been detected in codons 12, 13, 59, and 61. In vitro mutagenesis has also uncovered activating mutations in positions 63, 116, and 119 (2). Why these mutants have never been detected in vivo remains to be determined.

Increased expression of normal *ras* proto-oncogenes can also induce the transformed phenotype. Integration of a low number of copies of long terminal repeat (LTR)-linked normal *ras* genes or of multiple copies of the normal proto-oncogene also results in malignant transformation of NIH 3T3 cells (1). It should be noted that, even in the case of the oncogenes activated by point mutation, the degree of cellular transformation produced is a function of the amount of the mutated protein in the cells (1). Thus, the highly efficient expression of *ras* oncogenes in acutely transforming retroviruses is thought to account for their potent oncogenic potential. Also, a previously unnoticed point mutation in the last intron of the H-*ras* oncogene from T24 cells appears to be responsible for

increased expression and transforming activity of this oncogene (55).

Cooperation with other oncogenes

Although *ras* oncogenes can readily transform NIH 3T3 cells, they cannot transform primary fibroblasts. Transformation of primary fibroblasts by *ras* requires cooperation with immortalizing oncogenes such as c-*myc*, N-*myc*, E1A, or polyoma large T. In some cases *ras* oncogenes alone have been reported to transform primary cells, but only if strong transcriptional enhancers are driving their expression and the inhibitory effect of surrounding normal cells is eliminated (1). The nature of this inhibitory effect remains to be determined, although it can be overcome by treatment with phorbol esters or the use of strong retroviral promoters (1). These observations indicate that *ras* oncogenes can drive transformation but they are highly inefficient in overcoming senescence, and the establishment of malignant transformation by *ras* requires complementary changes involving immortalization (continuous proliferation) of the target cells. However, at least in some cases, establishment as continuous cell lines may not be sufficient for transformation by *ras*. Transformation of rat REF52 cells with *ras* oncogenes requires complementation with E1A or SV40 (56). Therefore, other requirements besides continuous proliferation may be needed for *ras* transformation.

Ras *oncogenes and human tumorigenesis*

Ras oncogenes have been detected in a great variety of human tumors, although their incidence varies considerably with tumor type. Activated *ras* oncogenes have been detected in 20 to 40% of colorectal tumors (2) and 95% of pancreatic carcinomas (57). In contrast, they are rarely found in breast cancer or stomach tumors (reviewed in 2). Although activation of N-*ras* shows some prevalence in the hematopoietic system and of K-*ras* in malignancies of epithelial origin (carcinomas), there is no strict correlation between a particular *ras* oncogene and a particular type of tumor. Also, the occurrence of activated *ras* genes does not correlate with any clinical or histopathological feature of the tumors. Significantly, the occurrence of *ras* oncogenes is not limited to malignant tumors, as benign tumors such as keratoacanthomas and polyps and adenomas of the intestine (2) have been described as carrying activated *ras* genes.

The activation of *ras* oncogenes is only a step in the multistep process of tumor development. An important question is whether *ras* activation is an early or late event during in vivo carcinogenesis. Animal models of induced carcinogenesis point strongly to a role of *ras* genes in initiation of neoplastic processes (reviewed in refs 1–3). In humans, the presence of mutated *ras* genes in premalignant tumors indicates that activation of *ras* may be an early event too. However, there are also reports of *ras* activation being detected late in the course of carcinogenesis (2). Whatever the timing of activation, there is increasing evidence that activation of *ras* oncogenes is not sufficient to trigger tumorigenesis, and secondary genetic

events are needed to convert the initiated cells to the malignant tumor stage (1-3). Support for this idea is provided by the presence of activated *ras* oncogenes in premalignant human and animal tumors (1-3) and by the delayed kinetics of tumor formation in transgenic mice (58) carrying activated *ras* oncogenes or in animals in which the *ras* oncogene is introduced through retroviral transduction of midgestation embryos (59).

Mammalian suppressors of ras *transformation*

There is ample evidence for the occurrence of mammalian genes able to revert morphological transformation and/or tumorigenicity caused by mutated *ras* alleles. Tumorigenicity is frequently suppressed in somatic cell hybrids between tumor cell lines and normal diploid cells (1). From this observation and the consistent detection of chromosome deletions in many tumors, the hypothesis emerged that suppressor genes (antioncogenes) may exist that are able to counteract the effect of transforming oncogenes. Genetic analysis of suppressed cellular hybrids and of tumorigenic segregants derived from them has permitted the assignment of putative suppressor genes to specific human and rodent chromosomes. Reversion achieved through direct introduction of these chromosomes into appropriate tumor cells has confirmed this view (1). In the case of *ras*-transformed cell lines and *ras*-derived tumors, intraspecific fusion of human or Chinese hamster cell lines carrying *ras* oncogenes with their untransformed normal counterparts results in hybrids that have lost the tumorigenicity although for the most part they retain the transformed morphology (reviewed in 1). This suggests that different genes are involved in suppression of tumorigenesis as opposed to suppression of morphological transformation alone.

The existence of genes that suppress morphological transformation but not tumorigenicity is supported by recent experiments in which transfection of normal human placental DNA into rat cells transformed by *ras* oncogenes resulted in revertant clones that had lost the morphology of transformed cells and required anchorage for growth but did not completely abolish tumorigenesis (60). The suppressor gene activity has bene cloned within an 18-kbp human DNA fragment and it will be interesting to determine the nature of the gene product(s) accomplishing this suppression.

Suppression of both transformed morphology and tumorigenicity was described years ago in flat revertants of the DT cell line, a clone of NIH 3T3 cells transformed by v-K-*ras* (1). These normal revertants expressed high levels of *ras* protein and had rescuable viruses but they could not be retransformed by K-*ras* or any other *ras* oncogene. Fusion experiments with cells transformed by other oncogenes indicated that this *ras*-suppressor phenotype also suppressed transformation by other oncogenes of the tyrosine kinase and serine/threonine kinase types. This is in agreement with the existence of common biochemical pathways for transformation discussed in a previous section.

In recent experiments, similar flat revertants from the DT cell line have been obtained by transfection with a

human cDNA library (18). Surprisingly, a cDNA possessing the revertant activity codes for a *ras*-like protein (Krev-1) that has the same amino acid sequence as the *ras*-related rap1 protein (16, 18). Mechanistically, this suggests that a ras-like protein competes with a *ras* oncogene in order to suppress its malignant effects.

The opposite situation, in which a *ras* mutant suppresses normal endogenous *ras* function, has also been described (35). The Asn17 mutation of H-*ras* appears to inhibit cell proliferation by competing with endogenous *ras*. Interestingly, NIH 3T3 cells transformed by *mos* and *raf*, but not *src*, were resistant to inhibition by Asn17. These results again support a participation of these oncogenes in common biochemical pathways transducing proliferation or differentiation signals.

FUTURE DEVELOPMENTS

The actual role of *ras* proteins in physiological and pathological processes will be understood only when we fully understand the biochemical and molecular mechanisms involving this increasingly complex collection of *ras* and *ras*-like gene products. The recent discovery of genes that suppress the transforming effects of *ras* alleles and the identification of other proteins that interact directly with *ras* proteins suggest that the next few years will see great advances in this direction. We hope that this process will culminate with the discovery of the actual effector molecule or molecules for all different *ras* proteins.

All this biochemical knowledge should allow us to determine exactly where the *ras* proteins interact with the complex cellular pathways transducing signals that control proliferation and/or differentiation. An improved understanding of the basic mechanisms controlling proliferation and differentiation should eventually be useful for management, diagnosis, or treatment of cancer. 𝔽𝕁

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